

1 **Antibody repertoire induced by SARS-CoV-2 spike protein immunogens**

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9 **One Sentence Summary:** SARS-CoV-2 Spike induced immune response

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21 **ABSTRACT**

22 Multiple vaccine candidates against SARS-CoV-2 based on viral spike protein are under  
23 development. However, there is limited information on the quality of antibody response generated  
24 following vaccination by these vaccine modalities. To better understand antibody response  
25 induced by spike protein-based vaccines, we immunized rabbits with various SARS-CoV-2 spike  
26 protein antigens: S-ectodomain (S1+S2) (aa 16-1213), which lacks the cytoplasmic and  
27 transmembrane domains (CT-TM), the S1 domain (aa 16-685), the receptor-binding domain  
28 (RBD) (aa 319-541), and the S2 domain (aa 686-1213 as control). Antibody response was analyzed  
29 by ELISA, Surface Plasmon Resonance (SPR) against different Spike proteins in native  
30 conformation, and a pseudovirion neutralization assay to measure the quality and function of the  
31 antibodies elicited by the different Spike antigens. All three antigens (S1+S2 ectodomain, S1  
32 domain, and RBD) generated strong neutralizing antibodies against SARS-CoV-2. Vaccination  
33 induced antibody repertoire was analyzed by SARS-CoV-2 spike Genome Fragment Phage  
34 Display Libraries (SARS-CoV-2 GFPDL), which identified immunodominant epitopes in the S1,  
35 S1-RBD and S2 domains. Furthermore, these analyses demonstrated that surprisingly the RBD  
36 immunogen elicited a higher antibody titer with 5-fold higher affinity antibodies to native spike  
37 antigens compared with other spike antigens. These findings may help guide rational vaccine  
38 design and facilitate development and evaluation of effective therapeutics and vaccines against  
39 COVID-19 disease.

40

## 41 INTRODUCTION

42 The ongoing pandemic of SARS-CoV-2 has resulted in more than 2 million human cases  
43 and 125,000 deaths as of 15<sup>th</sup> April 2020. Therefore, development of effective vaccines for  
44 prevention and medical countermeasures for treatment of SARS-CoV-2 infection is a high global  
45 priority. The spike glycoprotein has been identified as the key target for protective antibodies  
46 against both SARS-CoV-1 and SARS-CoV-2(1-4). Consequently, multiple versions of the SARS-  
47 CoV-2 spike proteins are currently under evaluation as vaccine candidates utilizing different  
48 modalities and delivery systems(5). However, only limited knowledge exists on antibody  
49 repertoire or quality of the immune response generated following vaccination by different spike  
50 vaccine antigens. Therefore, it is important to perform comprehensive evaluation of post-  
51 vaccination antibody response to elucidate the quality of the immune responses elicited by spike-  
52 based vaccine candidates to determine immune markers that may predict clinical benefit which  
53 can facilitate evaluation of vaccine candidates.

54 To better understand vaccination-induced antibody response, we immunized rabbits with  
55 several SARS-CoV-2 spike proteins: the S-ectodomain (S1+S2) (aa 16-1213) lacking the  
56 cytoplasmic and transmembrane domains (delta CT-TM), the S1 domain (aa 16-685), the receptor-  
57 binding domain (RBD) (aa 319-541), and the S2 domain (aa 686-1213), as a control. Post-  
58 vaccination sera were analyzed by Genome Fragment Phage Display Libraries covering the entire  
59 spike gene (SARS-CoV-2 GFPDL) to determine the polyclonal antibody epitope repertoire  
60 generated following vaccination as previously applied for other diseases(6-10). In addition, we  
61 employed several antibody binding assays (ELISA, Surface Plasmon Resonance (SPR) based real-  
62 time kinetics assay) (10-12) and an *in vitro* SARS-CoV-2 pseudovirion neutralization assay to  
63 measure the quality and function of the antibodies elicited by the different SARS-CoV-2 spike

64 antigens. This study could inform development and evaluation of SARS-CoV-2 vaccines and  
65 therapeutics based on the spike glycoprotein.

66

## 67 **RESULTS:**

### 68 **Rabbit immunization with SARS-CoV-2 Spike antigens**

69 Most spike-based vaccines currently under development are designed to contain the  
70 receptor-binding domain (RBD; aa 319-541) in some form. Therefore, we evaluated four different  
71 commercially available SARS-CoV-2 spike protein and subdomains: the Spike S1+S2 ectodomain  
72 (aa 16-1213), the S1 domain (aa 16-685), RBD domain (aa 319-541), and the S2 domain (aa 686-  
73 1213) as a control, which is devoid of RBD (Fig. 1A, Suppl. Fig. 1). These spike proteins were  
74 either produced in HEK 293 mammalian cells (S1 and RBD) or insect cells (S1+S2 ectodomain  
75 and S2 domain). The purified S1+S2 ectodomain, the S1 domain, and the RBD proteins retained  
76 the functional activity as demonstrated in SPR assay using human ACE2 protein, the SARS-CoV-  
77 2 receptor (Fig. 1B). The S1+S2 ectodomain, S1 domain and RBD (black, blue and red binding  
78 curves, respectively) demonstrated high-affinity interaction with human ACE2. The control S2  
79 domain protein (green curve), lacking the RBD, did not bind to human ACE2, demonstrating  
80 specificity of this receptor-binding assay (Fig.1B).

81 Female New Zealand white rabbits were immunized twice intra-muscularly at a 14-day  
82 interval with 50 µg of the purified proteins mixed with Emulsigen Adjuvant. Sera were collected  
83 before (pre-vaccination) and after the first and second vaccination and analyzed for binding  
84 antibodies in ELISA and SPR, in a pseudovirion neutralization assay, and by GFPDL analysis.

85

## 86 **Antibody Response following immunization with different Spike antigens**

87 Serial dilutions of post-second vaccination rabbit sera were evaluated for binding of serum  
88 IgG to various spike proteins and domains in ELISA (S1+S2; black, S1; blue, RBD; red, and S2;  
89 green) (Fig. 1C). Representative titration curves to spike ectodomain (S1+S2) and to the RBD in  
90 IgG-ELISA are shown in Suppl. Fig. 2. End-point titers of the serum IgG were determined as the  
91 reciprocal of the highest dilution providing an optical density (OD) twice that of the negative  
92 control (Fig. 1C). All four immunogens elicited strong IgG binding to the spike ectodomain  
93 (S1+S2). Binding to the individual domains (S1, S2, and RBD) was specific, in that sera generated  
94 by S2 vaccination bound to S2, but not to S1 or RBD, and vice-versa (Fig. 1C).

95 SPR allows following antibody binding to captured antigens in real-time kinetics, including  
96 total antibody binding in resonance units (Max RU) and affinity kinetics (Suppl. Fig. 3). In ELISA,  
97 the antigens directly coated in the wells can be partially denatured increasing the likelihood of  
98 presenting epitopes that are not seen on the native form of the protein by the polyclonal serum IgG.  
99 On the other hand, in our SPR, the purified recombinant spike proteins were captured to a Ni-NTA  
100 sensor chip to maintain the native conformation (as determined by ACE2 binding) to allow  
101 comparisons of binding to and dissociation from the four proteins. Importantly, the protein density  
102 captured on the chip surface is low (200 RU) and was optimized to measure primarily monovalent  
103 interactions, so as to measure the average affinity of antibody binding in the polyclonal serum (8,  
104 13). Additionally, while ELISA measured only IgG binding, in SPR, all antibody isotypes  
105 contributed to antibody binding to the captured spike antigen. In the current study, all rabbit sera  
106 contained anti spike antibodies that were at least 86% IgG (data not shown). Serial dilutions of  
107 post-vaccination serum were analyzed for binding kinetics with different spike proteins (Suppl.  
108 Fig. 3). The spike ectodomain (S1+S2) generated antibodies that predominantly bound to S1+S2

109 (black bar), followed by the S1 protein (blue bar), and 3-fold lower antibody binding to the RBD  
110 and the S2 domain (red and green bars, respectively) (Fig. 1D). The S1 domain antigen induced  
111 antibodies that bound with similar titers (Max RU values) to the S1+S2, S1 and RBD proteins  
112 (black, blue and red bars, respectively), and did not show reactivity to the S2 domain (green bar).  
113 However, the antibody reactivity of rabbit anti-S1 serum to S1+S2 domain was 3-fold lower than  
114 the antibodies in the rabbit anti-S1+S2 serum. RBD immunization generated similar high-titer  
115 antibody binding to S1+S2, S1 and RBD (black, blue and red bars, respectively), (Fig. 1D). In  
116 contrast, the S2 domain induced antibodies that primarily bound to homologous S2 antigen (green  
117 bars) and only weakly binding to the S1+S2 ectodomain (black bars), and no binding to either S1  
118 or RBD (Fig. 1D).

119         Antibody off-rate constants, which describe the fraction of antigen–antibody complexes  
120 that decay per second, were determined directly from the serum sample interaction with SARS-  
121 CoV-2 spike ectodomain (S1+S2), S1, S2, and RBD using SPR in the dissociation phase only for  
122 sensorgrams with Max RU in the range of 20–100 RU (Suppl. Fig. 3) and calculated using the  
123 BioRad ProteOn manager software for the heterogeneous sample model as described before(11).  
124 These off rates provide additional important information on the affinity of the antibodies following  
125 vaccination with the different spike proteins that are likely to have an impact on the antibody  
126 function *in vivo*, as was observed previously in studies with influenza virus, RSV and Ebola virus  
127 (13-15). Surprisingly, we observed significant differences in the affinities of antibodies elicited by  
128 the four spike antigens (Fig. 1E). Specifically, the RBD induced 5-fold higher affinity antibodies  
129 (slower dissociation rates) against S1+S2 (black), S1 (blue) and RBD (red) proteins, compared  
130 with the post-vaccination antibodies generated by other three immunogens (Fig. 1E).

131 SARS-CoV-2 neutralization was measured using SARS-CoV-2-FBLuc in a single-cycle  
132 PsVN assay in Vero E6 cells. The average percent inhibition by post-first and post-second rabbit  
133 vaccination are shown in Fig. 1F. Pre-vaccination rabbit sera (Control Rb) did not neutralize  
134 SARS-CoV-2 in PsVN assay. Sera generated by S1+S2-ectodomain, S1 and RBD (1:40 dilution)  
135 (but not anti-S2) showed 50-60% virus neutralization after a single vaccination, and 93-98% virus  
136 inhibition by the post-second vaccination sera (Fig. 1F).

137

### 138 **Epitope repertoires recognized by antibodies generated against SARS-CoV-2 spike antigens**

139 The constructed SARS-CoV-2 GFPDL contains sequences ranging from 50-1500 bp long  
140 from the spike gene (GenBank #MN908947) with  $>10^{7.2}$  unique phage clones. The SARS-CoV-2-  
141 GFPDL displayed linear and conformational epitopes with random distribution of size and  
142 sequence of inserts that spanned the entire spike gene. SARS-CoV-2 GFPDL panning with  
143 individual post-second vaccination rabbit sera were conducted as described in Methods. The  
144 numbers of IgG-bound SARS-CoV-2 GFPDL phage clones with different serum sample ranged  
145 between  $2.6 \times 10^4$  to  $9.8 \times 10^5$ /mL (Fig. 2A). Graphical distribution of representative clones with  
146 a frequency of  $\geq 2$ , obtained after affinity selection, and their alignment to the spike protein of  
147 SARS-CoV-2 are shown for the four vaccine groups (Fig. 2 B-E). The spike (S1+S2) ectodomain  
148 induced diverse antibody response that included strong binding to epitopes in the C-terminal region  
149 of the soluble protein spanning the HR2 region (i.e., multiple phage clones with similar inserts).  
150 This region may not be highly exposed on the virions or infected cells but is clearly immunogenic  
151 in the soluble recombinant spike ectodomain. In addition, the rabbit anti-S1+S2 antibodies bound  
152 diverse epitopes spanning the RBD and to a lesser degree to the N-terminal domain (NTD) and the  
153 C-terminal region of S1, and the N-terminus of S2, including the fusion peptide (Fig. 2B and Suppl.

154 Table 1). The S1 domain elicited very strong response against the C-terminal region of S1 protein  
155 and a diverse antibody repertoire recognizing the NTD and RBD/RBM regions (Fig. 2C and Suppl.  
156 Table 1). The recombinant RBD induced high-titer antibodies that were highly focused to the  
157 RBD/RBM (Fig. 2E, and Suppl. Table 1). In contrast, the recombinant S2 immunogen after two  
158 immunizations in rabbits elicited antibodies primarily targeting the C-terminus of the S2 protein  
159 (CD-HR2).

160 All the immunodominant antigenic sites identified by the SARS-CoV-2 GFPDL panning  
161 of all 4 immune sera on the spike sequence are shown in Suppl. Fig. 4. Alignment of the sequence  
162 with other coronaviruses shows that some of the antigenic sites are >70% conserved among several  
163 coronavirus strains isolated from humans and bats, especially those located in the S2 domain  
164 (Suppl. Table 1). Structural depiction of these antigenic sites on the SARS-CoV-2 spike (Suppl.  
165 Fig. 5; in blue on PDB#6VSB), demonstrated that most of these antigenic sites identified in the  
166 current study are surface exposed on the native prefusion spike(2).

167

## 168 **DISCUSSION**

169 In this study, we performed an in-depth evaluation of antibody response generated by  
170 various SARS-CoV-2 spike antigens that are similar to the vaccine antigens being used in clinical  
171 development(5, 16, 17). Bioinformatics approach previously identified 279 potential B-cell  
172 epitopes and 48 potential T cell epitopes in the Spike glycoproteins of SARS-CoV viruses, based  
173 on human antibody responses to the SARS-CoV-1 infection and the corresponding epitopes in  
174 SARS-CoV-2 spike (Grifoni et al. Table 4) (18). We compared the predominant antigenic sites  
175 identified by antibodies in our study generated by different spike antigens with the B cell epitopes  
176 predicted by Grifoni *et al.*(18). Four of the predicted B epitopes overlapped with the sequences we



177 identified in our GFPDL analysis: aa 287-317 in NTD-RBD overlaps with our antigenic site aa  
178 298-363 which is 77% homologous between SARS-CoV-1 and SARS-CoV-2; aa 524-598 and aa  
179 601-640, in the C-terminus of S1 overlap with our antigenic site containing aa 548-632 (78.8 %  
180 conservation between SARS-CoV-1 and SARS-CoV-2); aa 802-819 in the S2 domain/FP overlaps  
181 with our antigenic site aa 768-828 (83% conserved between SARS-CoV-1 and SARS-CoV-2) (Fig.  
182 S4 and Suppl. Table 1). The other epitopes identified in our study cover less conserved sequences  
183 between the two SARS-CoV viruses that are unique to the SARS-CoV-2 spike and were not  
184 identified in the *in-silico* approach by Grifoni et al.

185 Surprisingly, the S2 domain doesn't appear to elicit as many neutralizing antibodies as  
186 RBD or S1. Although S2 contains the fusion peptide, it does not appear to be as immunogenic,  
187 compared with S1 or RBD, in generating binding antibodies to the intact spike (S1+S2)  
188 ectodomain, as observed in both IgG ELISA and SPR. Even though we characterized the purified  
189 proteins in various assays, there is a possibility that the structure of the antigens used in the study  
190 is different from the corresponding authentic spike protein on the surface of SARS-CoV-2 virion  
191 particle.

192 One unexpected finding in this study was the higher affinity of antibodies elicited by the  
193 RBD compared with the other spike antigens (S1+S2 ectodomain, S1 and S2 domains). In earlier  
194 studies, with vaccines against H7N9 avian influenza we found important correlation between  
195 antibody affinity against the hemagglutinin HA1 globular domain and control of virus loads after  
196 challenge of ferrets with H7N9 (19). In study of patients recovering from Zika virus (ZIKV)  
197 infections, their antibody affinity against ZIKV E-DIII correlated with lower clinical scores(20).  
198 In a large randomised clinical trial of IVIG hyper-enriched for influenza virus antibodies (hIVIG),  
199 in adults hospitalised with confirmed influenza A or B infections, a statistically significant

200 virological benefit and clinical benefit for patients infected with B strains, directly correlated with  
201 stronger antibody affinities of the hIVIG for circulating B strains (14). In a recent longitudinal  
202 study of Ebola virus disease survivor, affinity maturation to Ebola virus GP was associated with a  
203 rapid decline in viral replication and illness severity in this patient (13). Thus, vaccines that can  
204 elicit high affinity antibodies may have a significant advantage for *in-vivo* clinical outcome of  
205 SARS-CoV-2 infection and contribute to amelioration of disease in infected individuals.  
206 Therefore, in addition to measurements of antibody-binding titers and virus neutralization, this and  
207 the previous studies demonstrate the importance of assessments of antibody affinity maturation  
208 during SARS-CoV-2 vaccine trials.

209 In summary, our study highlights the need to perform comprehensive analysis of immune  
210 response generated following vaccination or SARS-CoV-2 infection to identify biomarkers of  
211 protective immunity. In-depth understanding of quantitative and qualitative aspects of immune  
212 responses generated by different spike protein vaccine antigens could aid the development and  
213 evaluation of effective SARS-CoV-2 therapeutics and vaccines.

214

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225 **AUTHOR CONTRIBUTIONS:**

226 **Designed research:** S.K.

227 **Performed research:** S.R., J. T., E.C., L.K., G. G., S.L., T.W., and S.K.

228 **Conducted Animal study:** L.K., G. G., and S.K.

229 **SARS-CoV-2 neutralization assays:** S.L., and T.W.

230 **Contributed to Writing:** H.G. and S.K.

231

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233

234 **Materials & Correspondence.** Correspondence and material requests should be addressed to the  
235 corresponding author (S.K.).

236

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238 Care and Use Committee (IACUC) under Protocol #2008-10. The animal care and use protocol  
239 meets National Institutes of Health guidelines.

240

241 **FIGURE LEGENDS**

242 **Figure 1: SARS-CoV-2 spike binding and SARS-CoV-2 neutralization by serum antibodies**  
243 **generated following rabbit immunization with spike antigens.** A) Schematic representation of  
244 the SARS-CoV-2 spike protein and subdomains. Spike S1+S2 ectodomain (aa 16-1213) lacks the  
245 cytoplasmic and transmembrane domains (CT-TM), S1 domain (aa 16-685), RBD domain (aa 319-  
246 541), and S2 domain (aa 686-1213), all containing 6x His tag at C-terminus, were produced in  
247 either HEK 293 mammalian cells (S1 and RBD) or insect cells (S1+S2 ectodomain and S2  
248 domain). (B) Binding of purified proteins to human ACE2 proteins in SPR. Sensorgrams represent  
249 binding of purified spike proteins on His-captured chips to 5  $\mu\text{g/mL}$  human ACE2 protein. (C)  
250 Anti-spike reactivity of post-immunization rabbit sera. Serial dilutions of post-second vaccination  
251 rabbit sera were evaluated for binding to various spike proteins and domains (S1+S2; black, S1;  
252 blue, RBD; red, and S2; green) in ELISA. Representative titration curves are shown in Fig. S2.  
253 End-point titers of the serum samples were determined as the reciprocal of the highest dilution  
254 providing an optical density (OD) twice that of the negative control (no serum was used as negative  
255 control). (D) SPR binding of antibodies from rabbits immunized twice with SARS-CoV-2 antigens  
256 to spike protein and domains from SARS-CoV-2 (S1+S2; black, S1; blue, RBD; red, and S2;  
257 green). Total antibody binding is represented in resonance units in this figure for 10-fold serum  
258 dilution. All ELISA and SPR experiments were performed twice and the researchers performing  
259 the assay were blinded to sample identity. The variations for duplicate runs of ELISA and SPR  
260 were  $<8\%$  and  $<5\%$ , respectively. The data shown are average values of two experimental runs.  
261 (E) Antibody off-rate constants, which describe the fraction of antigen-antibody complexes that  
262 decay per second, were determined directly from the serum/ sample interaction with SARS-CoV-  
263 2 spike ectodomain (S1+S2), S1, S2, and RBD using SPR in the dissociation phase only for the

264 sensorgrams with Max RU in the range of 20–100 RU. (F) Virus neutralization titers were  
265 measured against SARS-CoV-2-FBLuc in a single-round pseudovirus neutralization assay in  
266 triplicates (see Methods). The average percentage inhibition after the first and second vaccination  
267 (1:40 serum dilution) for each group are shown. Pre-vaccination rabbit sera also did not neutralize  
268 in PsVN assay (Control Rb).

269

270 **Figure 2: Antibody repertoires generated by different SARS-CoV-2 spike antigens.** (A)  
271 Number of IgG-bound SARS-CoV-2 GFPDL phage clones using the post-second vaccination  
272 rabbit polyclonal sera from the vaccine groups in Fig 1. (B-E) Graphical distribution of  
273 representative clones with a frequency of  $\geq 2$ , obtained after affinity selection, and their alignment  
274 to the Spike protein of SARS-CoV-2 are shown for the four vaccine groups: S1+S2 ectodomain  
275 (B), S1 (C), S2 domain (D) and S1-Receptor binding domain (RBD) (E). The thickness of each  
276 bar represents the frequency of repetitively isolated phage, with the scale shown enclosed in a red  
277 box in the respective alignments in each panel. The GFPDL affinity selection data was performed  
278 twice. Similar numbers of phage clones and epitope repertoire were observed in both phage display  
279 analyses.

280

## 281 SUPPLEMENTAL INFORMATION

282

283 **Figure S1:** Purified SARS-CoV-2 proteins analyzed by SDS-PAGE under reducing and non-  
284 reducing conditions.

285

286 **Figure S2:** Anti-spike reactivity of post-vaccination rabbit sera in ELISA.

287

288 **Figure S3:** Steady-state equilibrium analysis of different dilutions of serum antibodies binding to

289 Spike protein by SPR.

290

291 **Figure S4.** Sequence alignment of spike protein from diverse CoV strains.

292

293 **Figure S5.** Structural representation of antigenic sites identified in SARS-CoV-2 using GFPDL

294 analyses.

295

296 **Supplementary Table 1.** Sequence similarity (%) of SARS-CoV-2 spike antigenic sites with other

297 CoV strains.

298

299 **METHODS:**

300

301 **Recombinant CoV Proteins**

302           Recombinant SARS-CoV-2 proteins were purchased from Sino Biologicals (S1+S2  
303 ectodomain; 40589-V08B1, S1; 40591-V08H, RBD; 40592-V08H or S2; 40590-V08B).  
304 Recombinant purified proteins used in the study were either produced in HEK 293 mammalian  
305 cells (S1 and RBD) or insect cells (S1+S2 ectodomain and S2 domain).

306

307 **Rabbit immunization Studies**

308           Female New Zealand white rabbits (Charles River labs) were immunized twice intra-  
309 muscularly at 14-days interval with 50 µg of purified proteins mixed with Emulsigen Adjuvant.  
310 Sera were collected before (pre-vaccination) and after 1<sup>st</sup> and 2<sup>nd</sup> vaccination and analyzed for  
311 binding antibodies in ELISA, SPR, neutralization assay and GFPDL analysis.

312

313 **ELISA**

314           96 well Immulon plates were coated with 100 ng/100 µL of recombinant spike protein and  
315 protein domains in PBS overnight at 4°C. Starting at a 1:100 dilution, serum samples were serially  
316 diluted 1:5 and applied to the protein-coated plate in 10 µL for 1 hr at ambient temperature. Serum  
317 samples were assayed in duplicate. Naïve serum samples were assayed along with the experimental  
318 samples. After three washes with PBS/0.05% Tween 20, bound antibodies were detected with a  
319 donkey anti-rabbit IgG Fc-specific HRP-conjugated antibody (Jackson Immuno Research) After

320 1hr, plates were washed as before and OPD was added for 10min. Absorbance was measured at  
321 492 nm. End titer was determined as 2-fold above the average of the absorbance values of the  
322 naïve serum samples. The end titer is reported as the last serum dilution that was above this cutoff.

323

### 324 **Antibody binding kinetics of post-vaccination sera to recombinant SARS-CoV-2 proteins by** 325 **Surface Plasmon Resonance (SPR)**

326 Steady-state equilibrium binding of post-vaccination rabbit polyclonal serum was  
327 monitored at 25°C using a ProteOn surface plasmon resonance (BioRad). The purified  
328 recombinant Spike proteins were captured to a Ni-NTA sensor chip with 200 resonance units (RU)  
329 in the test flow channels. The native functional activity of the Spike proteins was determined by  
330 binding to the 5 µg/mL human ACE2 protein.

331 For serum analysis, the protein density on the chip was optimized such as to measure  
332 monovalent interactions independent of the antibody isotype. Serially diluted (10-, 20-, 40-, 80-,  
333 and 160-fold of freshly prepared sera were injected at a flow rate of 50 µl/min (120 sec contact  
334 duration) for association, and disassociation was performed over a 600-second interval. Responses  
335 from the protein surface were corrected for the response from a mock surface and for responses  
336 from a buffer-only injection. SPR was performed with serially diluted serum of each animal in this  
337 study. Antibody isotype analysis for the SARS-CoV-2 spike protein bound antibodies in the  
338 polyclonal serum was performed using SPR. Total antibody binding was calculated with BioRad  
339 ProteOn manager software (version 3.1). All SPR experiments were performed twice and the  
340 researchers performing the assay were blinded to sample identity. In these optimized SPR  
341 conditions, the variation for each sample in duplicate SPR runs was <5%. The maximum resonance



342 units (Max RU) data shown in the figures was the RU signal for the 10-fold diluted serum sample.  
343 Antibody off-rate constants, which describe the fraction of antigen–antibody complexes that decay  
344 per second, are determined directly from the serum/ sample interaction with SARS CoV-2 spike  
345 ectodomain (S1+S2), S1, S2, and RBD using SPR in the dissociation phase only for the  
346 sensorgrams with Max RU in the range of 20–100 RU and calculated using the BioRad ProteOn  
347 manager software for the heterogeneous sample model as described before(11). Off-rate constants  
348 were determined from two independent SPR runs.

349

### 350 **SARS-CoV-2 pseudovirus production and neutralization assay**

351 Human codon-optimized cDNA encoding SARS-CoV-2 S glycoprotein (NC\_045512) was  
352 synthesized by GenScript and cloned into eukaryotic cell expression vector pcDNA 3.1 between  
353 the BamHI and XhoI sites. Pseudovirions were produced by co-transfection Lenti-X 293T cells  
354 with pMLV-gag-pol, pFBluc, and pcDNA 3.1 SARS-CoV-2 S using Lipofectamine 3000. The  
355 supernatants were harvested at 48h and 72h post transfection and filtered through 0.45-mm  
356 membranes.

357 For neutralization assay, 50  $\mu$ L of SARS-CoV-2 S pseudovirions were pre-incubated with  
358 an equal volume of medium containing serum at varying dilutions at room temperature for 1 h,  
359 then virus-antibody mixtures were added to Vero E6 cells in a 96-well plate. After a 3 h incubation,  
360 the inoculum was replaced with fresh medium. Cells were lysed 48 h later, and luciferase activity  
361 was measured using luciferin-containing substrate.

362

### 363 **Gene Fragment Phage Display Library (GFPDL) construction**

364 SARS-CoV-2 spike gene was chemically synthesized and used for cloning and  
365 construction of phage display libraries. A gIII display-based phage vector, fSK-9-3, was used  
366 where the desired polypeptide can be displayed on the surface of the phage as a gIII-fusion protein.  
367 Purified DNA containing spike gene was digested with *DNase I* to obtain gene fragments of 100-  
368 1000 bp size range and used for GFPDL construction as described previously (6-8). The phage  
369 libraries constructed from the SARS-CoV-2 spike gene display viral protein segments ranging in  
370 size from 30 to 350 amino acids, as fusion protein on the surface of bacteriophage.

371

#### 372 **Affinity selection of SARS-CoV-2 GFPDL phages with polyclonal rabbit serum**

373 Prior to panning of GFPDL with polyclonal serum antibodies, serum components that  
374 could non-specifically interact with phage proteins were removed by incubation with UV-killed  
375 M13K07 phage-coated petri dishes (8). Equal volumes of each post-vaccination rabbit serum were  
376 used for GFPDL panning. GFPDL affinity selection was carried out in-solution with protein A/G  
377 (IgG) specific affinity resin as previously described (6, 7, 9) Briefly, the individual rabbit serum  
378 was incubated with the GFPDL and the protein A/G resin, the unbound phages were removed by  
379 PBST (PBS containing 0.1 % Tween-20) wash followed by washes with PBS. Bound phages were  
380 eluted by addition of 0.1 N Gly-HCl pH 2.2 and neutralized by adding 8  $\mu$ L of 2 M Tris solution  
381 per 100  $\mu$ L eluate. After panning, antibody-bound phage clones were amplified, the inserts were  
382 sequenced, and the sequences were aligned to the SARS-CoV-2 spike gene, to define the fine  
383 epitope specificity in the post-vaccination rabbit sera. The GFPDL affinity selection data was  
384 performed blindly in a blinded fashion. Similar numbers of bound phage clones and epitope  
385 repertoire were observed in the two GFPDL panning.

386

387 **Data Availability**

388 The datasets generated during and/or analyzed during the current study are available from the  
389 corresponding author on reasonable request.

390

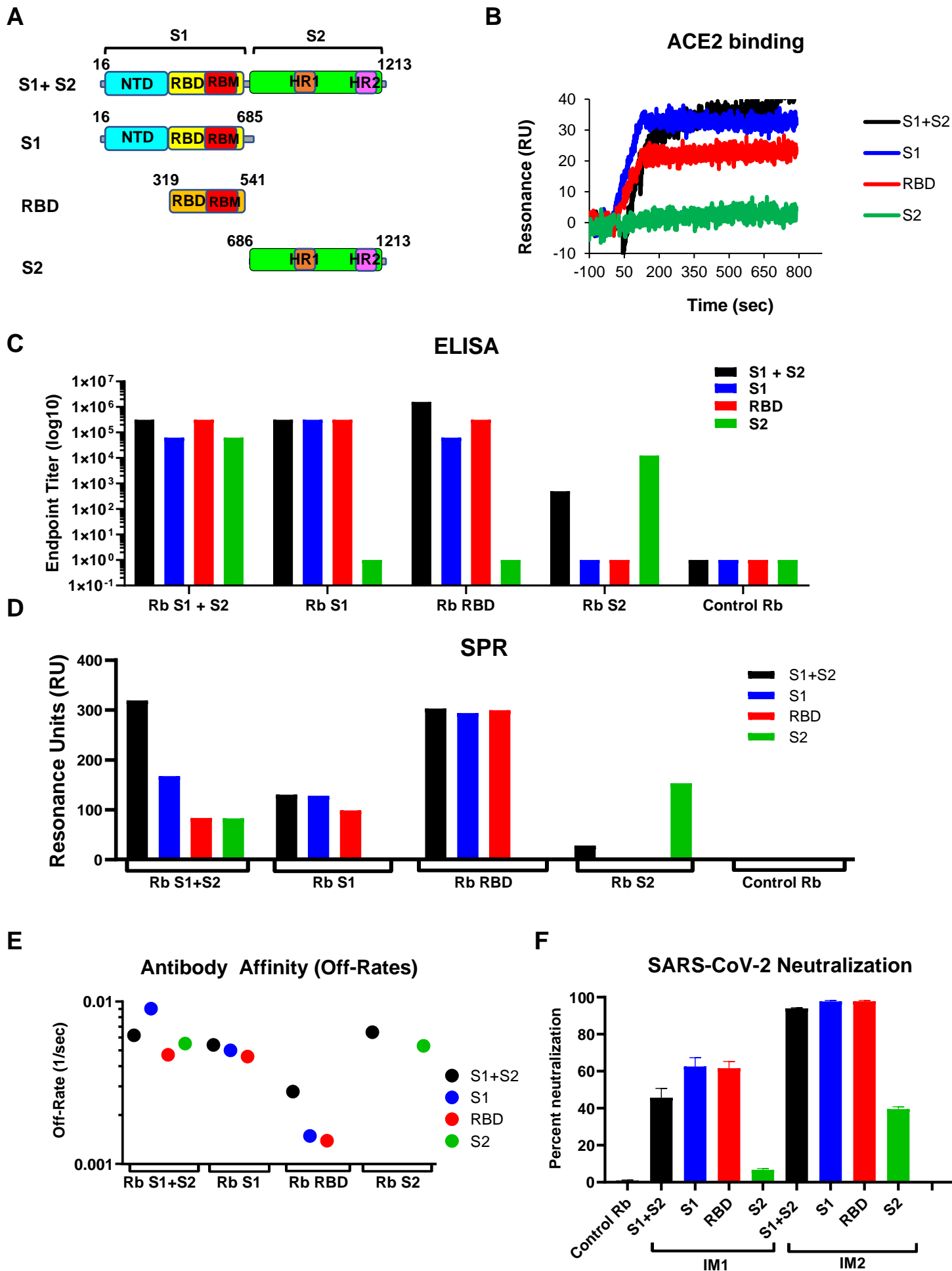
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459

# Fig 1



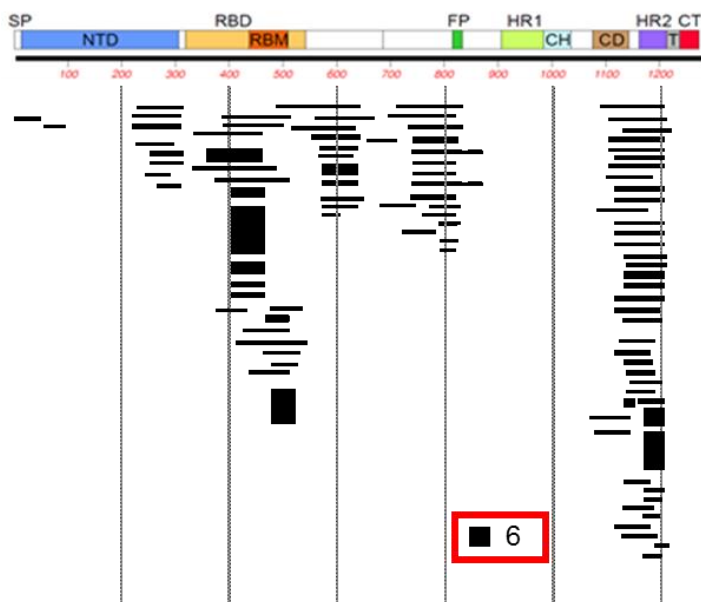
# Fig 2

**A**

	Rb S1+S2	Rb S1	Rb S2	Rb RBD	Control Rb
IgG	5.1E+04	2.2E+05	2.63E+04	9.88E+05	4.20E+01

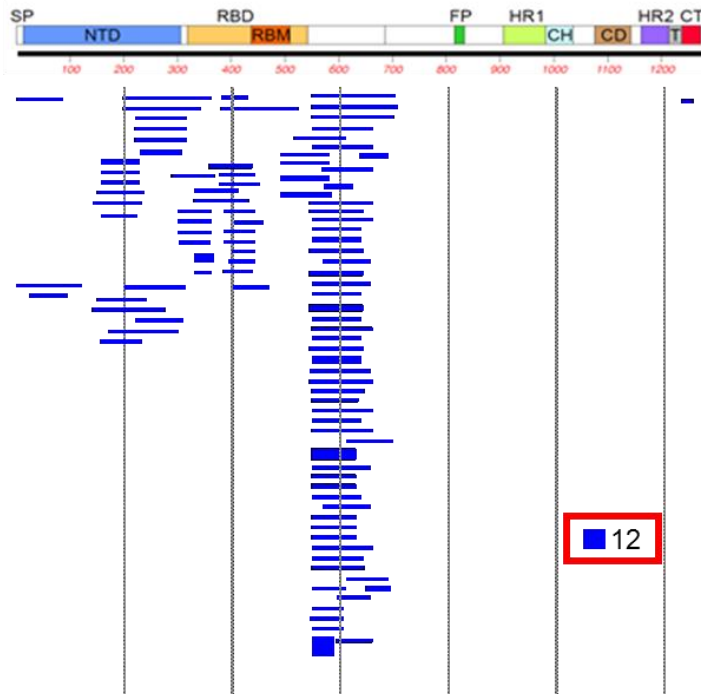
**B**

## S1 + S2 domain (aa 16-1213)



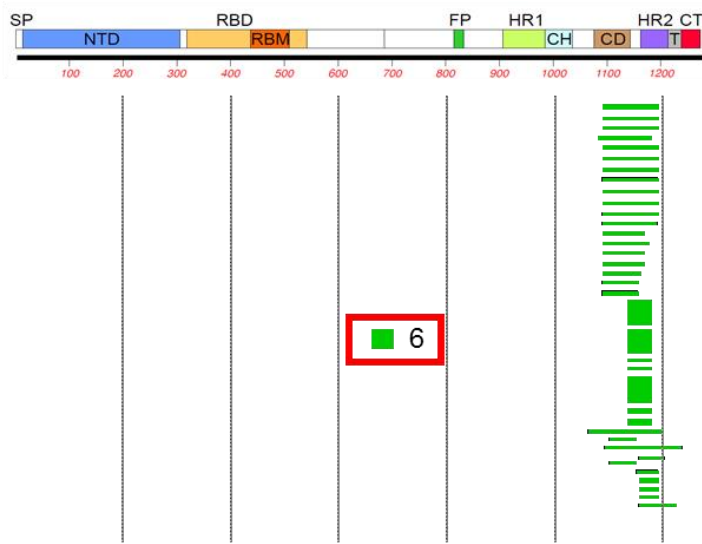
**C**

## S1 domain (aa 16-685)



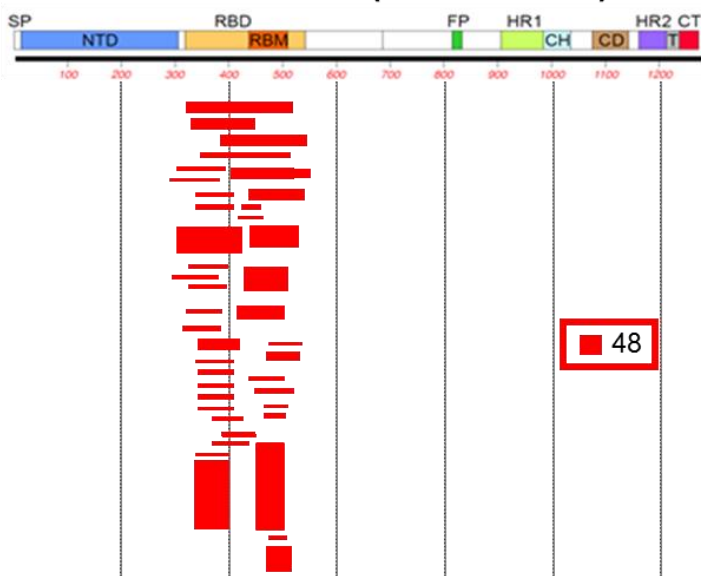
**D**

## S2 domain (aa 319-541)



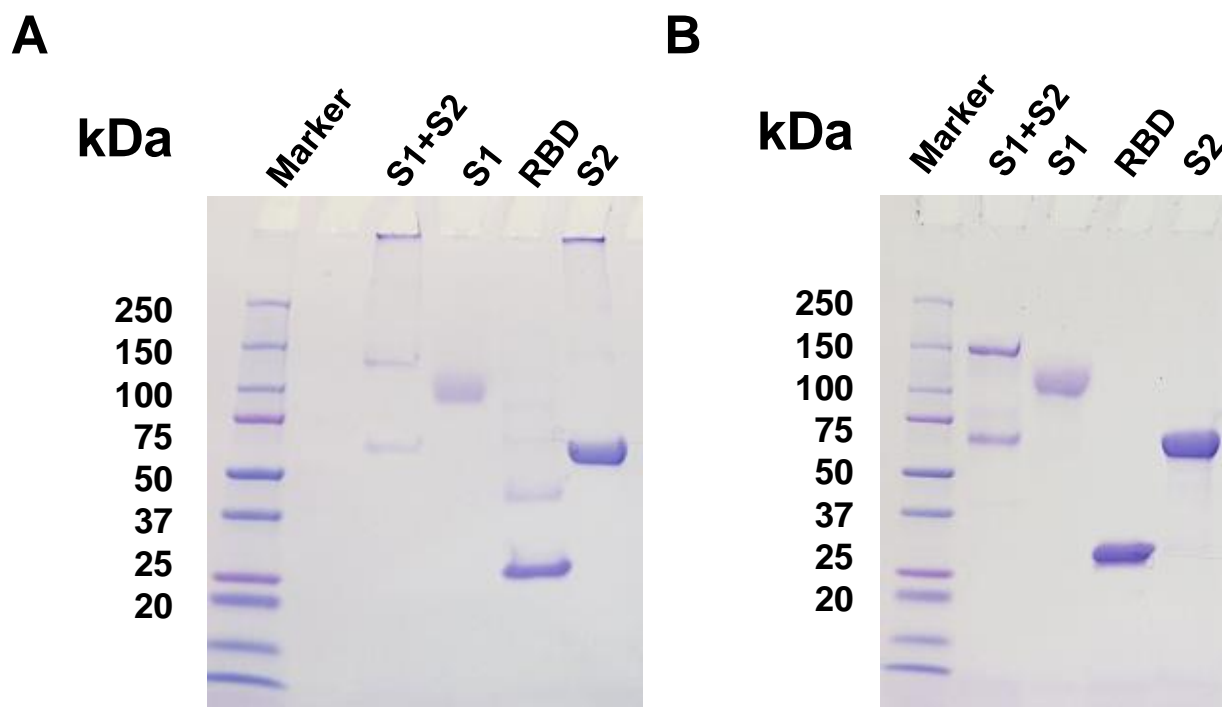
**E**

## RBD (aa 686-1213)



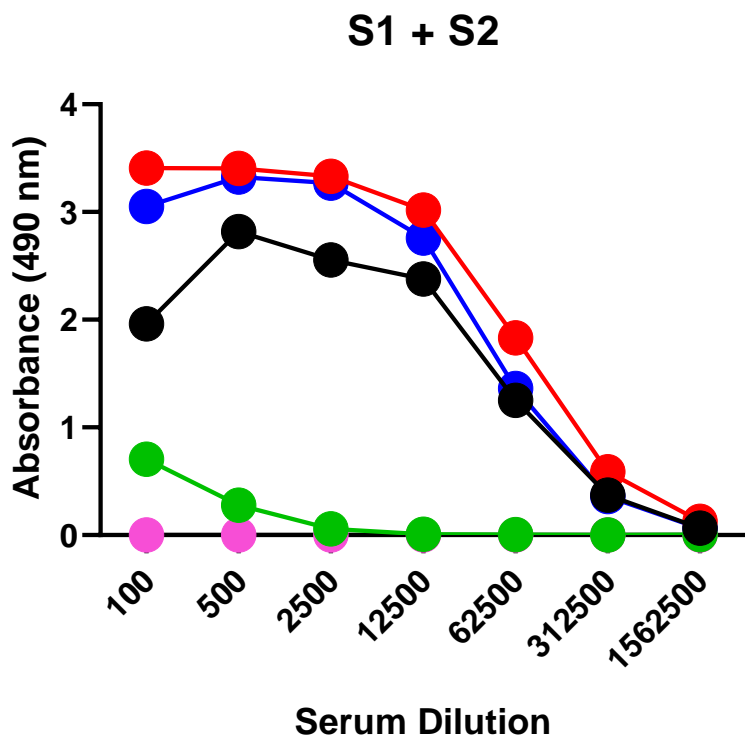




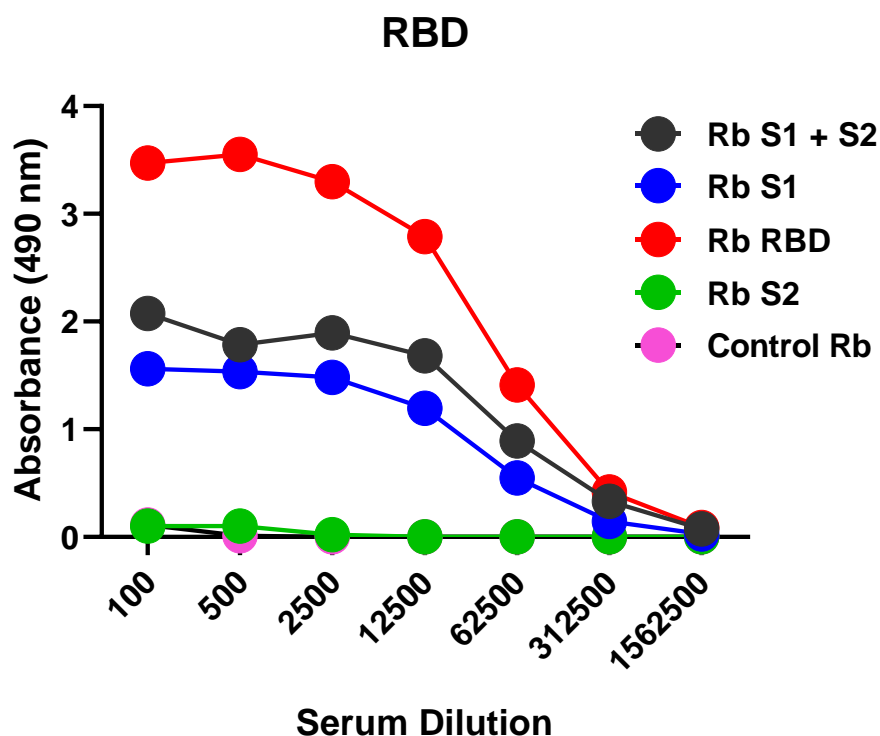


**Supplementary Figure 1: Purified SARS-CoV-2 proteins analyzed by SDS-PAGE and under reducing and non-reducing conditions. Related to figure 1.** 2  $\mu$ g of purified proteins was run in SDS-PAGE under non-reducing (A) and reducing (B) conditions. The gels were stained with Coomassie blue.

**A**

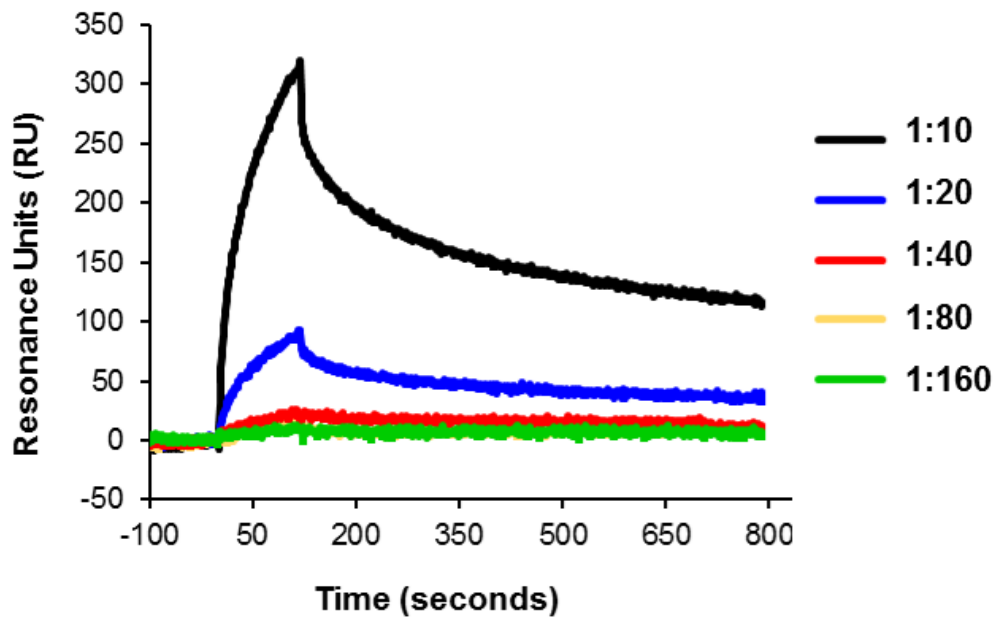


**B**



**Supplementary Figure 2: Anti-Spike reactivity of post-vaccination rabbit sera in ELISA. Related to figure 1.** Post-vaccination rabbit sera following two immunizations with different SARS-CoV-2 spike vaccine antigens (S1+S2; black, S1; blue, RBD; red, S2; green and pre-vaccination control; pink) in ELISA. Average antibody binding to recombinant Spike (S1+S2) ectodomain (A) and S1-RBD (B) is shown in ELISA using HRP-conjugated goat anti-rabbit IgG specific antibody.

## Rb S1+ S2



### Supplementary Figure 3

**Steady-state equilibrium analysis of serum antibodies binding by SPR. Related to figure 1.** Serial dilutions of post-2<sup>nd</sup> vaccination rabbit antiserum against SARS-CoV-2 Spike (S1+S2 ectodomain) were injected simultaneously onto SARS-CoV-1 S1+S2 captured on a Ni-NTA sensor chip and on a surface free of protein (used as a blank). Binding responses from the protein surface were corrected for the response from the mock surface and for responses from a separate, buffer only injection. Unvaccinated Rabbit control sample at 10-fold dilution did not show any binding in SPR.

**Supplementary Figure 4. Sequence alignment of Spike protein from diverse CoV strains. Related to figures 1 & 2.** An alignment of the spike proteins of SARS-CoV-2 (Genbank#MN908947), SARS-CoV-1 BJ01 strain (Genbank#AAP30030.1), MERS CoV KOR/KNIH/2015(Genbank#AKN11075.1), Bat SARS-like CoV ZC45 (Genbank#AVP78031.1), Bat SARS-like CoV ZXC21 (Genban#AVP78042.1), Bat CoV BM48-31/BGR/2008 (Genbank#ADK66841.1), Human CoV 2c EMC/2012 (Genbank#AFS88936.1), Human CoV NL63 (NCBI#YP\_003767.1), and Human CoV HKU1 (NCBI#YP\_173238.1) was performed using Clustal W multiple alignment application. Various domains of the spike protein are the S1 subunit (AA 1-685), RBD (AA 319-541), FP (816-834) and S2 (686-1273) subunits. The SARS-CoV-2 antigenic regions/sites discovered in this study using the post-vaccination antibodies with different SARS-CoV-2 vaccine antigens are depicted above the SARS-CoV-2 spike protein sequence in alternating black and grey lines with the corresponding AA residues for visualization.



540 550 560

SARS CoV-2 GCVIAWNSNN LDSRVGGNYN YLYRLFR --- KSNLKPFFERD  
SARS CoV-1 BJ01 GCVLAWNTRN IDATSTGNYN YKYRYLR --- HGKLRPFERD  
MERS CoV KOR/2015 TCLILATVPH NLTTITKPLK YSYINKC --- SRLLSDDRTE  
Bat SL CoV ZC45 GCVIAWNTAK QD--VG--N YFYRSHR --- STKLKPFERD  
Bat SL CoV ZXC21 GCVIAWNTAK QD--TG--H YFYRSHR --- STKLKPFERD  
Bat CoV BM48-31/2008 GCVIAWNTNS LDSSNE--- FFYRRFR --- HGKIKPYGRD  
Human CoV 2c EMC/2015 TCLILATVPH NLTTITKPLK YSYINKC --- SRLLSDDRTE  
Human CoV NL63 ETYVALPIYY QHTDINFAT ASFGGSC --- YVCKPHQVN  
Human CoV HKU1 SCQLYSLPA INVTINNYNP SSWNRRYGFN NFNLSHSHVV YSRYCFVSNV TFCPCAKPSF ASSCKSHKPP

478-516

570 580 590 600 610 620 630

SARS CoV-2 ISTEIYQAGS TPCNGVEGFN CY----- FPLQSYGF QPTN----- GVG YQPYRVVVLS  
SARS CoV-1 BJ01 ISNVPFSPDG KPCTPPALN- CY----- WPLNDYGF YTTT----- GIG YQPYRVVVLS  
MERS CoV KOR/2015 VPQLVNAVQY SPCVSTVPST VWEDGDYRQ QLSPLEGGGW LVAS----- GST VAMTEQLQMG  
Bat SL CoV ZC45 LSSDE----- NGVR----- TLSTYDF NPNV----- PLE YQATRVVVLS  
Bat SL CoV ZXC21 LSSDE----- NGVR----- TLSTYDF NPNV----- PLE YQATRVVVLS  
Bat CoV BM48-31/2008 LSNVLENPST GTCSAEGLN- CY----- KPLASVGF TQSS----- GIG YQPYRVVVLS  
Human CoV 2c EMC/2015 VPQLVNAVQY SPCVSTVPST VWEDGDYRQ QLSPLEGGGW LVAS----- GST VAMTEQLQMG  
Human CoV NL63 ISLNGNTSVC VRTSHFSIRY IYN----- RVKSGSP GDSS----- WHI YLKSGETPFS  
Human CoV HKU1 SASCPIGTNY RSCESTTVLD HTDWCRCSC LPDPITAYDP RSCSQKSLV GVGHECAGFG VDEERCGVLD

640 650 660 670 680 690 700

SARS CoV-2 FELLHAPATV CGPKKSTNLV KN----- KCVNFMFNG  
SARS CoV-1 BJ01 FELLNAPATV CGPKLSTDLI KN----- QCVNFMFNG  
MERS CoV KOR/2015 FGITVQYGTD TNSVCPKLEF ANDTKIASQL GNCVEYSLYG  
Bat SL CoV ZC45 FELLNAPATV CGPKLSTQLV KN----- QCVNFMFNG  
Bat SL CoV ZXC21 FELLNAPATV CGPKLSTQLV KN----- QCVNFMFNG  
Bat CoV BM48-31/2008 FELLNAPATV CGPKQSTELV KN----- KCVNFMFNG  
Human CoV 2c EMC/2015 FGITVQYGTD TNSVCPKLEF ANDTKIASQL GNCVEYSLYG  
Human CoV NL63 FSKLNNFQKF KTCFSTVEV PG----SCNF PLEATWHYTS  
Human CoV HKU1 GSYNVSC LCS TDAFLGWSYD TCVSNNRCNI FSNFILNGIN SGTTCNDLL QPN--TEVFT DVCVDYDLYG

548-590

574-636

710 720 730 740 750 760 770

SARS CoV-2 LTGTGVLTES NKKFLP-FQQ FGRDIADTTD AVRDPQTELEI LDITPCSFGG VSVITPGTNT SNQVAVLYQD  
SARS CoV-1 BJ01 LTGTGVLTPS SKRFQF-FQQ FGRDVSDFTD SVRDPKTSEI LDISPFSFGG VSVITPGTNA SSEVAVLYQD  
MERS CoV KOR/2015 VSGRGVFNQC TAVGVR-QQH FVYDAYQNLV GYYS-DDGNY YCLRACVSVV VSVIYD--KE TKTHATLFGS  
Bat SL CoV ZC45 LKGTGVLTD SSKRFQS-FQQ FGKASDFID SVRDPQTELEI LDITPCSFGG VSVITPGTNT SLEVAVLYQD  
Bat SL CoV ZXC21 LKGTGVLTD SSKRFQS-FQQ FGKASDFID SVRDPQTELEI LDITPCSFGG VSVITPGTNT SLEVAVLYQD  
Bat CoV BM48-31/2008 LTGTGVLTPS TKKRFQF-FQQ FGRDVSDFTD SVRDPKTSEI LDIAPCSYGG VSVITPGTNA SSEVAVLYQD  
Human CoV 2c EMC/2015 VSGRGVFNQC TAVGVR-QQH FVYDAYQNLV GYYS-DDGNY YCLRACVSVV VSVIYD--KE TKTHATLFGS  
Human CoV NL63 YTIIVGALYVT WSEGMS-ITG VPPVSG-IR EFSNLVNLNC TKYNIYDYVG TGIIRSSNQS LAGGITVYSN  
Human CoV HKU1 ITGQGIKKEV SAVYNSWQN LLYDSNGNII GFKDFVTNKT YNIFPCYAGR VSAAFH--QN ASSLALLYRN

548-632

780 790 800 810 820 830 840

SARS CoV-2 VNCTEVEVAI HADQLTPTWR VYSTGS--NV FQTRAGCLIG AEHV--NNSY ECDIPIGAGI CASYQTQNS  
SARS CoV-1 BJ01 VNCTDVSTAI HADQLTPAWR IYSTGN--NV FQTQAGCLIG AEHV--DTSY ECDIPIGAGI CASYHTVS--  
MERS CoV KOR/2015 VACEHISSTM SQYSRSTRM LKRRDSTYGP LQTPVGCVLG LVNSS-LFVE DCKLPLGQSL CALPDTFSTL  
Bat SL CoV ZC45 VNCTDVPTTI HADQLTPAWR IYATGT--NV FQTQAGCLIG AEHV--NASY ECDIPIGAGI CASYHTAS--  
Bat SL CoV ZXC21 VNCTDVPTTI HADQLTPAWR IYAIGT--SV FQTQAGCLIG AEHV--NASY ECDIPIGAGI CASYHTAS--  
Bat CoV BM48-31/2008 VNCTDVPTML HADQISHDWR VYAFRNDGNI FQTQAGCLIG AAYD--NSSY ECDIPIGAGI CAKYTNVSS--  
Human CoV 2c EMC/2015 VACEHISSTM SQYSRSTRM LKRRDSTYGP LQTPVGCVLG LVNSS-LFVE DCKLPLGQSL CALPDTFSTL  
Human CoV NL63 SGNLLGFKNV STGNIFIVTP CNQPDQVAVY QQSIIIGAMTA VNESRYGLQN LLQLPNFYVY SNGGNNCTTA  
Human CoV HKU1 LKCSYVLNNI SLTTQP---- FDSYLGCVFN ADNLTDYSVS SCALRMGSGF CVDYNSPSSS

850 860 870 880 890 900 910

SARS CoV-2 P---RRARS VASQSIIAYT MSLGAENSVA YSNN---SIA IPTNFTISVT TEILPVSMTK TSVDCMTMYIC  
SARS CoV-1 BJ01 -----LLRS TSQKSIVAYT MSLGADSSIA YSNN---TIA IPTNFSISIT TEVMPVSMAR TSVDCNMYIC  
MERS CoV KOR/2015 T---PRSVR SVPGEMRLAS IAFNHPIQVD QLNSYFKLS IPTNFSFGVT QEYIQTTIQK VTDCKQYVC  
Bat SL CoV ZC45 -----ILRS TGQKAIWAYT MSLGAENSIA YANN---SIA IPTNFSISVT TEVMPVSMAR TSVDCMTMYIC  
Bat SL CoV ZXC21 -----ILRS TGQKAIWAYT MSLGAENSIA YANN---SIA IPTNFSISVT TEVMPVSMAR TSVDCMTMYIC  
Bat CoV BM48-31/2008 -----TLVR SGGHSILAYT MSLGDNQDIV YSNN---TIA IPMNFISIVT TEVLPVSMTRK TSVDCNMYIC  
Human CoV 2c EMC/2015 T---PRSVR SVPGEMRLAS IAFNHPIQVD QLNSYFKLS IPTNFSFGVT QEYIQTTIQK VTDCKQYVC  
Human CoV NL63 VMTYSNFGIC ADGSLIPVRP RNSSDNGISA IITAN---LS IPSNWTTSVQ VEYLQITSTP IVDVDCATYVC  
Human CoV HKU1 SRRRKRRSIS ASYRFVTFEP FNVSVFVNSI ESVGGLYEIK IPTNFTIVGQ EEFIQTNSPK VTIDCSLFVC

768-828

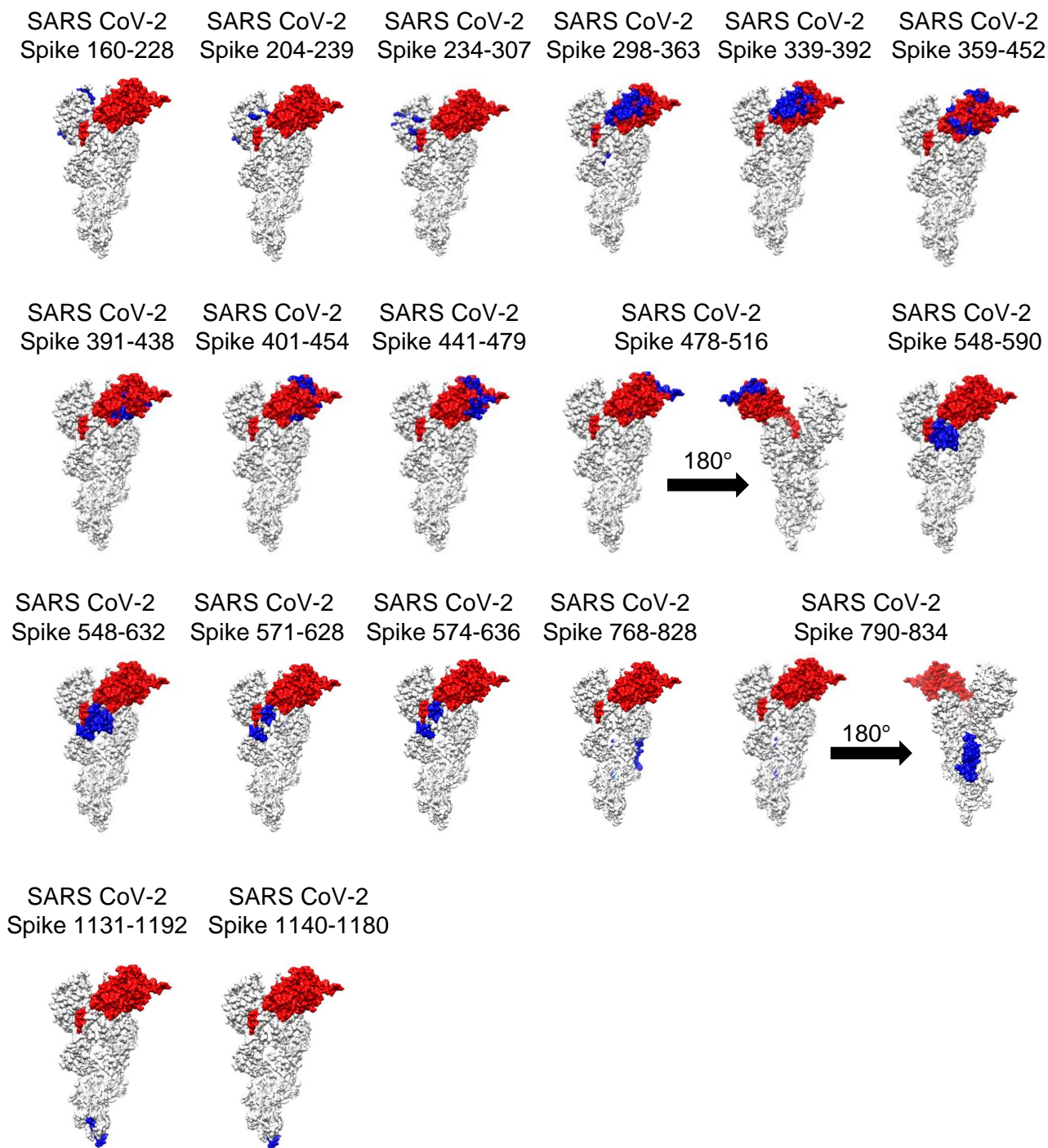
920 930 940 950 960 970 980

SARS CoV-2 GDSTECSNLL LQYGSFCTQL NRALTGIAVE QDKNTQEVFA QVKQIYKTPP IKDFGG-FNF SQILPDPSPK  
SARS CoV-1 BJ01 GDSTECANLL LQYGSFCTQL NRALSGIAAE QDRNTRVFA QVKQMYKTPT LKYFGG-FNF SQILPDELKP  
MERS CoV KOR/2015 NGFQKCEQLL REYGFQCSKI NQALHGANLR QDSDVRNLFA SVKSSQSSPI IPGFGGDFNL TLLEPVSIST  
Bat SL CoV ZC45 GDSIECSNLL LQYGSFCTQL NRALSGIAIE QDKNTQEVFA QVKQIYKTPP IKDFGG-FNF SQILPDPSPK  
Bat SL CoV ZXC21 GDSIECSNLL LQYGSFCTQL NRALSGIAIE QDKNTQEVFA QVKQIYKTPP IKDFGG-FNF SQILPDPSPK  
Bat CoV BM48-31/2008 GDSTECSNLL LQYGSFCTQL NRALAGIAVE QDRNTRDVA QTKAMYKTPS LKDFGG-FNF SQILPDPKAP  
Human CoV 2c EMC/2015 NGFQKCEQLL REYGFQCSKI NQALHGANLR QDSDVRNLFA SVKSSQSSPI IPGFGGDFNL TLLEPVSIST  
Human CoV NL63 NGNPRCKLL KQYTSACKTI EDALRLSAHL ETNDVSSMLT FDSNAFSLAN VTSFGD-YNL SSVLPQRNIR  
Human CoV HKU1 SNYAACHDLL SEYGTFACTNI NSILDEVNGL LDTTQLHVAD TLMQGVTLSS NLNTNLHFVD DNINFKSLIVG



```
.....|.....| .....|.....|
SARS CoV-2          K-FDEDDSEP VLKGVKLHYT
SARS CoV-1 BJ01    K-FDEDDSEP VLKGVKLHYT
MERS CoV KOR/2015  DRYEEYDLEP HKVHVH----
Bat SL CoV ZC45    K-FDEDDSEP VLKGVKLHYT
Bat SL CoV ZXC21   K-FDEDDSEP VLKGVKLHYT
Bat CoV BM48-31/2008 K-FDEDHSEP VLTGVKLHYT
Human CoV 2c EMC/2015 DRYEEYDLEP HKVHVH----
Human CoV NL63     DCGSTKLPYY EFEKVHVQ--
Human CoV HKU1     DEYGGHHDFFV IKTSHDD---
```





**Supplementary Figure 5. Structural representation of antigenic sites identified in SARS-CoV-2 using GFDL. Related to figure 2.** Antigenic sites identified using GFDL have been depicted in blue on surface structures of a monomer of PDB#6VSB (Wrapp et al., 2020) with a single receptor-binding domain (RBD) in the up conformation, wherever available using UCSF Chimera software. The RBD region is shaded in red (residues 319-541) on every structure. Those structures (SARS CoV-2 Spike 478-516 and 790-834), whose sites were not visible on the side depicted by flipping the structure by 180°.

**Table S1: Sequence conservation of Antigenic regions/sites among different CoV strains**

AA	Similarity of SARS CoV 2 antigenic sites to other CoV strains (%)								
	SARS CoV-2	SARS CoV-1	MERS	Bat SL CoV ZC45	Bat SL CoV ZXC21	Bat CoV BM48	Human CoV 2c	Human NL63	Human HKU1
160-228	100	53.6	9.7	62.3	60.8	39.1	9.7	12.6	5.7
204-239	100	55.5	10.5	63.8	63.8	44.4	10.5	21	8.1
234-307	100	52.7	32.4	74.3	71.6	48.6	32.4	6.4	27
298-363	100	77.2	30.3	80.3	80.3	78.7	30.3	7.5	30.3
339-392	100	87	24	75.9	75.9	83.6	24	3.6	27
359-452	100	80.8	20.2	74.4	72.3	76.8	20.2	5.2	20.2
391-438	100	83.3	20.8	83.3	83.3	81.2	20.8	8.3	22.9
401-454	100	74	16.6	72.2	68.5	70.3	16.6	5.5	12.9
441-479	100	46.1	10.2	41	35.8	30.7	10.2	10.2	2.7
478-516	100	61.5	14.2	43.5	43.5	56.4	14.2	12.5	8.7
548-590	100	74.4	16.2	74.4	74.4	79	16.2	4.6	22.7
548-632	100	78.8	17.6	80	80	75.2	17.6	4.7	22
574-636	100	80.9	14.2	87.3	87.3	76.1	14.2	3.1	22.2
768-828	100	83.6	41.5	96.7	96.7	80.3	41.5	24.6	24.2
790-834	100	86.6	46.9	100	100	84.4	46.9	32	31.4
1140-1180	100	100	26.1	97.5	97.5	75.6	26.1	14.5	33.3
1173-1207	100	100	45.7	97.1	97.1	82.8	45.7	20	37.1

\* Percent sequence conservation of GFPDL identified antigenic sites in the SARS CoV-2 spike protein (Genbank#MN908947), with CoV Spike proteins of SARS CoV-1 BJ01 strain (Genbank#AAP30030.1), MERS CoV KOR/KNIH/2015(Genbank#AKN11075.1), Bat SARS-like CoV ZC45 (Genbank#AVP78031.1), Bat SARS-like CoV ZXC21 (Genban#AVP78042.1), Bat CoV BM48-31/BGR/2008 (Genbank#ADK66841.1), Human CoV 2c EMC/2012 (Genbank# AFS88936.1), Human CoV NL63 (NCBI#YP\_003767.1), and Human CoV HKU1 (NCBI#YP\_173238.1) was calculated using Sequence Identity Matrix function in BioEdit.